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Characterization of *Toxoplasma gondii* isolates in free-range chickens from Chile, South America

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Abstract

The prevalence of *Toxoplasma gondii* in free-ranging chickens is a good indicator of the prevalence of *T. gondii* oocysts in the soil because chickens feed from the ground. The prevalence of *T. gondii* in 85 free-range chickens (*Gallus domesticus*) from Chile was determined. Antibodies to *T. gondii* were assayed by the modified agglutination test (MAT), and found in 47 of 85 (55.3.9%) chickens with titers of 1:5 in six, 1:10 in four, 1:20 in four 1: 40 in three, 1: 80 in nine, 1: 160 in four 1:320 in nine, and 1: 640 or higher in eight. Hearts and brains of 47 chickens with titers of 1:5 or higher were pooled for each chicken and bioassayed in mice. Tissues from 16 seronegative (MAT $<$ 1:5) chickens were pooled and fed to one *T. gondii*-free cat. Feces of the cat were examined for oocysts but none was found based on bioassay of fecal floats in mice. Hearts and brains from seven seronegative ($<$1:5) were pooled and bioassayed in mice; *T. gondii* was not isolated. *T. gondii* was isolated by bioassay in mice from 22 chickens with MAT titers of 1:20 or higher. Genotyping of these 22 isolates using polymorphisms at the loci SAG1, SAG2, SAG3, BTUB and GRA6 revealed three genotypes. Seventeen isolates had type II alleles and four isolates had type III alleles at all loci. One isolate contained the combination of type I and III alleles. This is the first report of genetic characterization of *T. gondii* isolates from Chile, South America.

Keywords: *Toxoplasma gondii*; Chickens; *Gallus domesticus*; Free-range; Chile; South America; Genotype

1. Introduction

Toxoplasma gondii infections are widely prevalent in human beings and animals worldwide (Dubey and Beattie, 1988). Humans become infected post-natally by ingesting tissue cysts from undercooked meat, consuming food or drink contaminated with oocysts,

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or by accidentally ingesting oocysts from the environment. However, only a small percentage of exposed adult humans develop clinical signs. It is unknown whether the severity of toxoplasmosis in immunocompetent persons is due to the parasite strain, host variability, or to other factors.

T. gondii isolates have been classified into three genetic types (I, II, III) based on restriction fragment length polymorphism (RFLP) (Ajzenberg et al., 2002a, 2002b, 2004; Aspinall et al., 2003; Boothroyd and Grigg, 2002; Dubey et al., 2004a,d; da Silva et al., 2005; Ferreira et al., 2004, 2006; Fuentes et al., 2001; Grigg et al., 2001; Howe and Sibley, 1995; Howe et al., 1997; Jungersen et al., 2002; Mondragon et al., 1998; Owen and Trees, 1999). The parasite was previously considered clonal with very low genetic variability. However, most of the information was derived from isolates from Europe and North America. Using newer markers for genetic characterization and using recently isolated strains from Brazil and French Guiana, higher genetic variability was revealed than previously reported (Ajzenberg et al., 2004; Lehmann et al., 2004).

We have initiated a worldwide study of *T. gondii* population structure. For this we have chosen the free-range chicken as the indicator host for soil contamination with *T. gondii* oocysts because they feed from the ground. Thus far, we have characterized strains from South America (Brazil (Dubey et al., 2002, 2003a,d, 2006b), Peru (Dubey et al., 2004b), Venezuela (Dubey et al., 2005f), Argentina (Dubey et al., 2003e, 2005c), Colombia (Dubey et al., 2005i), Central America and the Caribbean (Guatemala (Dubey et al., 2005b), Grenada, West Indies (Dubey et al., 2005e), Costa Rica (Dubey et al., in press), North America (USA (Dubey et al., 2003c; Lehmann et al., 2003), Mexico (Dubey et al., 2004c)), Africa and Middle East (Egypt (Dubey et al., 2003b), Israel (Dubey et al., 2004e), Mali, Kenya, Burkina Faso, and Democratic Republic of Congo (Dubey et al., 2005a)), Asia (Sri Lanka (Dubey et al., 2005d), India (Sreekumar et al., 2003)), and Europe (Austria (Dubey et al., 2005g), and Portugal (Dubey et al., 2006a)). These studies are still not complete, nevertheless, a pattern is emerging that isolates from Brazil are genetically distinct (Lehmann et al., 2004).

In the present paper, we attempted to isolate and genotype *T. gondii* from chickens from Chile, South America.

2. Materials and methods

2.1. Naturally infected chickens

Chickens ($n = 85$) were obtained from free-range chickens in rural farms from 85 different properties that were at least 500 m apart. They were purchased in four (A–D) batches in June to November 2005 (Table 1). Samples of brain, whole heart, and blood were collected from each chicken, and kept at 4 °C until sent by air to Beltsville, MD. Three to eleven days elapsed between killing of chickens and receipt of samples at Beltsville. Chickens from batches A, and C were badly autolysed when received at Beltsville, MD.

Table 1
Summary of chickens from Chile used for isolation of *T. gondii*

Batch number (experiment)	Chickens				Bioassay for *T. gondii*	
	Month 2005 received	Number of chickens	MAT titer		Number of seropositive chickens bioassayed in mice ≥1:5	Number of chickens positive
			<1:5	≥1:5		
A (Tx 189)	June	26	9	17 (14)[a]	17	4
B (Tx 192)	August	28	16[b]	12 (9)	12	5
C (Tx 205)	October	16	6	10 (8)	10	5
D (Tx 209)	November	15	7[c]	8 (8)	8	8
Total		85	28	47 (39)	47	22

[a] Number of chickens with titers of 1:20 or higher.
[b] Sixteen chicken tissues were fed to a cat.
[c] Seven chicken tissues were bioassayed in mice.

2.2. Serological examination

Sera of chickens were tested for *T. gondii* antibodies using eight dilutions, from 1:5 to 1: 640 with the modified agglutination test (MAT) as described by Dubey and Desmonts (1987).

2.3. Bioassay of chickens for *T. gondii* infection

Tissues of 70 of 85 chickens were bioassayed for *T. gondii* infection. Brains, and hearts of 47 chickens with titers of 1:5 were bioassayed individually in outbred female Swiss Webster (SW) mice obtained from Taconic Farms, Germantown, New York, as described (Dubey et al., 2002). In addition tissues of seven seronegative (MAT < 1:5) chickens from batch D were pooled and bioassayed in mice. Tissues were homogenized, digested in acidic pepsin, washed, and homogenate inoculated subcutaneously into five mice (Dubey, 1998).

Brains and hearts from 16 chickens from batch B (Table 1) with MAT titers of <1:5 were pooled and fed to one *T. gondii*-free cat (Dubey et al., 2002). Feces of the cat were examined for shedding of *T. gondii* oocysts 3–14 days post-ingesting chicken tissues as previously described (Dubey, 1995). Fecal floats were incubated in 2% sulfuric acid for 1 week at room temperature on a shaker to allow sporulation of oocysts and were bioassayed orally in mice (Dubey and Beattie, 1988). Tissue imprints of lungs and brains of mice that died were examined for *T. gondii* tachyzoites or tissue cysts. Survivors were bled on day 41 post-inoculation (p.i.) and a 1:25 dilution of serum from each mouse was tested for *T. gondii* antibodies with the MAT. Mice were killed 47 or 48 days p.i. and brains of all mice were examined for tissue cysts as described (Dubey and Beattie, 1988). The inoculated mice were considered infected with *T. gondii* when tachyzoites or tissue cysts were found in tissues.

2.4. Genetic characterization for *T. gondii*

T. gondii DNA was extracted from the tissues of all infected mice from each group (Table 1) and strain typing was performed using genetic markers SAG1, SAG2, SAG3, BTUB and GRA6 as described previously (Grigg et al., 2001; Howe et al., 1997; Khan et al., 2005) with modification (Table 2). In brief, the target DNA sequences were first amplified by multiplex PCR using external primers for all five markers. The reaction was carried out in 25 μl of volume containing 1 × PCR buffer, 2 mM $MgCl_2$, 200 μM each of the dNTPs, 0.15 μM each of the forward and reverse primers, 0.5 units of FastStart DNA polymerase (Roche Applied Science, Indianapolis, IN) and 1.5 μl of DNA extract. The reaction mixture was treated at 95 °C for 4 min, followed by 20 cycles of 94 °C for 30 s, 55 °C for 1 min and 72 °C for 2 min. Multiplex PCR amplified products (1.5 μl) were then used for nested PCR amplification (35 cycles) with internal primers for each marker separately, using an annealing temperature of 60 °C in 25 μl volume reaction mixture as above. To reveal the RFLP pattern of each reference strain, 3 μl of PCR

Table 2
Summary for genetic markers

Markers	External primers (for multiplex PCR)*	Internal primers (for nested PCR)	Restriction enzymes	Reference
SAG1	F: GTTCTAACCACGCACCCTGAG R: AAGAGTGGGAGGCTCTGTGA	F: CAATGTGCACCTGTAGGAAGC R: GTGGTTCTCCGTCGGTGTGAG	Sau96I + HaeII (double digest)	Grigg et al. (2001). **This study**
5′-SAG2	F: GCTACCTCGAACAGGAACAC R: GCATCAACAGTCTTCGTTGC	F: GAAATGTTTCAGGTTGCTGC R: GCAAGAGCGAACTTGAACAC	Sau3AI	Howe et al. (1997)
3′-SAG2	F: TCTGTTCTCCGAAGTGACTCC R: TCAAAGCGTGCATTATCGC	F: ATTCTCATGCCTCCGCTTC R: AACGTTTCACGAAGGCACAC	HhaI	Howe et al. (1997)
SAG3	F: CAACTCTCACCATTCCACCC R: GCGCGTTGTTAGACAAGACA	F: TCTTGTCGGGTGTTCACTCA R: CACAAGGAGACCGAGAAGGA	NciI	Grigg et al. (2001)
BTUB	F: TCCAAAATGAGAGAAATCGT R: AAATTGAAATGACGGAAGAA	F: GAGGTCATCTCGGACGAACA R: TTGTAGGAACACCCGGACGC	BsiEI + TaqI (double digest)	Khan et al. (2005). **This study**
GRA6	F: ATTTGTGTTTCCGAGCAGGT R: GCACCTTCGCTTGTGGTT	F: TTTCCGAGCAGGTGACCT R: TCGCCGAAGAGTTGACATAG	MseI	Khan et al. (2005)

* F = forward primer; R = reverse primer.

Table 3
Isolation of *T gondii* from tissues of seropositive chickens from Chile

Chickens					Isolation in mice	Genetic characterization					
Batch and chicken number	Farmhold location	Longitude	Latitude	MAT titer	No. infected[a]	Isolate ID	SAG1	SAG2	SAG3	BTUB	GRA6
A											
4	Manquehue	72° 56′ 497775″	38° 43′ 208552″	>1280	4[b]	TgCkCh1	I	III	III	III	III
6	Manquehue	73° 16′ 812423″	38° 37′ 226948″	40	5	TgCkCh2	II or III	II	II	II	II
10	Manquehue	73° 17′ 46111″	38° 37′ 149264″	80	5[c]	TgCkCh3	II or III	III	III	III	III
26	Teodoro Smith	73° 17′ 344046″	38° 38′ 218206″	>640	4	TgCkCh4	II or III	II	II	II	II
B											
1	Chol-Chol -1	73° 21′ 221413″	38° 38′ 251843	20	1	TgCkCh5	II or III	II	II	II	II
9	Nueva Imperial	73° 18′ 56 9153″	38° 38′ 219354″	>640	5	TgCkCh6	II or III	II	II	II	II
14	Labranza	73° 18′ 251945″	38° 40′ 275402″	320	5	TgCkCh7	II or III	II	II	II	II
19	Niagara	73° 16′ 376925″	38° 39′ 565804″	160	5	TgCkCh8	II or III	II	II	II	II
20	Niagara	73° 17′ 474438″	38° 39′ 389511″	320	5	TgCkCh9	II or III	II	II	II	II
C											
1	Manquehue	73° 2′ 356223″	38° 59′ 34389″	>640	3	TgCkCh10	II or III	II	II	II	II
2	Manquehue	72° 44′ 256777″	38° 44′ 47049″	80	1	TgCkCh11	II	II	II	II	II
4	Manquehue	72° 40′ 394367″	38° 46′ 541131″	160	2	TgCkCh12	II or III	II	II	II	II
11	Manquehue	72° 41′ 483548″	38° 47′ 256607″	>640	5	TgCkCh13	II or III	II	II	II	II
15	Manquehue	72° 41′ 252368″	38° 48′ 309734″	>640	5	TgCkCh14	II or III	II	II	II	II
D											
6	Trovolhue	72° 40′ 103952″	38° 49′ 105921″	160	5	TgCkCh15	II or III	III	III	III	III
7	Trovolhue	72° 39′ 527846″	38° 48′ 268118″	320	4	TgCkCh16	II or III	II	II	II	II
9	Trovolhue	72° 40′ 34,248″	38° 47′ 433754″	80	5	TgCkCh17	II or III	II	II	II	II
10	Trovolhue	72° 39′ 168576″	38° 47 072283″	320	5	TgCkCh18	II or III	III	III	III	III
12	Trovolhue	72° 38′ 286306″	38° 47′ 298957″	80	5	TgCkCh19	II or III	II	II	II	II
13	Trovolhue	72° 33′ 390036″	38° 45′ 178909″	320	5	TgCkCh20	II or III	III	III	III	III
14	Trovolhue	72° 32′ 173923″	38° 45′ 504332	320	5	TgCkCh21	II or III	II	II	II	II
15	Trovolhue	72° 49′ 487978″	38° 37′ 42452″	80	5	TgCkCh22	II or III	II	II	II	II

[a] Of five mice inoculated.
[b] Three mice died on days 17, 24, and 27.
[c] One mouse died on day 39.

products were mixed with 17 μl of digestion reaction containing 1 × NEB reaction buffer, 0.1 mg/ml BSA and 1 unit of restriction enzyme. The reaction was carried out by incubating at the proper temperature for each restriction enzyme by the manufacturer's instruction (New England BioLab, Beverly, MA). For markers SAG1 and BTUB, two restriction enzymes were used simultaneously (double digest) in one reaction mixture. The digested PCR products were resolved in a 2.5% agarose gel by electrophoresis in the presence of 0.3 μg/ml ethidium bromide and visualized under UV light.

3. Results

Antibodies to *T. gondii* were found in 47 of 85 (55.3%) chickens with titers of 1:5 in six, 1:10 in four, 1:20 in four 1: 40 in three, 1: 80 in nine, 1: 160 in four 1:320 in nine, and 1: 640 or higher in eight.

T. gondii was isolated from tissues of 22 of 37 chickens with MAT titers of 1:20 or higher (Table 2); from one of four with a titer of 1:20, from one of four with a titer of 1:40, from five of nine with a titer of 1:80, from one of four with a titer of 1:160, and 12 of 20 with titers of 1:320 or higher (Table 3). Only four of 94 mice infected with *T. gondii* died. Although 94 of 110 mice inoculated with tissues of infected chickens acquired toxoplasmosis very few tissue cysts were found in the brains of infected mice.

The cat fed tissues from seronegative chickens did not shed oocysts.

The *T. gondii* isolates obtained by bioassay in mice were designated TgCkCh 1-22 (Table 3).

Genotyping of these 22 isolates using polymorphisms at the SAG1, SAG2, SAG3, BTUB, and GRA6 loci revealed that 17 isolates had type II alleles and four isolates had type III alleles at all loci. One isolate contained the combination of type I and III alleles. All infected mice from each group had identical genotype; mixed infections were not found (Table 3).

4. Discussion

In the present study *T. gondii* was isolated by bioassay in mice from 22 of 37 (70%) chickens with titers of 1:20 or higher and not from 48 chickens with titers of 1:10 or less. Data from this and other studies with chickens (see Dubey et al., 2005c) are being accumulated for the validity of MAT for the detection of *T. gondii* in chickens.

Phenotypically and genetically, *T. gondii* isolates from chickens from Chile were like isolates from North America and Grenada, West Indies and different from Brazil, Colombia, Argentina, and Peru from South America. Most isolates from chickens from Brazil and Columbia were lethal for mice whereas isolates from North America and from Grenada did not kill inoculated mice. Genetically, none of *T. gondii* isolates from Colombia and Brazil was SAG2 Type II, whereas most isolates from chickens from Chile, North America and Grenada were Type II (Dubey et al., 2003c; Lehmann et al., 2003). The *T. gondii* isolates from Argentina were of variable pathogenicity to mice, and all three genetic types (I,II,III) were present (Dubey et al., 2003e, 2005c).These differences among *T. gondii* isolates from the four neighboring countries in South America (Argentina, Chile, Peru, and Brazil) are of interest. This is the first report of genetic characterization of *T. gondii* isolates from Chile

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